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Original Article

EFFECT OF ALPHA-AMYLASE PRODUCED BY *BACILLUS SUBTILIS ON* MAIZE KERNEL STARCH

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ABSTRACT

Bacillus subtilis was studied for extracellular α-amylase production. Bacillus subtilis has been reported as one of the important α-amylase producer. Hence, attempt has been made to study the growth profile and enzyme production by the bacteria. Growth activity was analyzed and enzyme activity was calculated by using Iodine assay and DNSA assay. The yield of α-amylase reached 11g/100 ml at 28h growth and started declining after that due to low glucose concentration in the medium. Growth for liquefying action showed the maximum enzyme production and 35U/ml was achieved at 72h cultivation and was found to decline after that. It is reported that α-amylase production in lower volume does not reduce to the extent as in higher volume as 100ml. The reasons have been discussed during the work. It was observed that maximum alpha amylase production by Bacillus subtilis occurred when cell population reached the peak. The activity of α-amylase enzyme was observed on maize starch hydrolysis. The enzyme preparation had affinity towards maize starch granule and was almost completely absorbed onto it. Maltose was released as the main end product on hydrolysis of raw maize starch. A strain Bacillus subtilis, produced α amylase with characteristics suitability for application in maize starch processing.

Keywords: *α amylase, Bacillus subtilis, growth curve, maize starch hydrolysis*

INTRODUCTION

Enzymes as biocatalyst are capable to hydrolyse the various chemical substances. One of them is maize starch. Enzymes are secreted by living organisms and now a day's some bacteria are becoming the most popular host for enzyme production, due to their consideration as GRAS (Generally Regarded as Safe). *Bacillus subtilis* is fast and easy growing bacteria even in simple and cheaper media, secreted enzymes directly into the nutrient broth medium. This reduces the cost of production and purification. So in the present work we have selected this bacterium for their growth study and alpha amylase enzyme production study. To avoid sporulation, batch culture system was used for the present research work. We need to also show the Units as produced in a volume broth.

So far attempts to use ***Bacillus subtilis*** have emphasized mainly on the extracellular alpha amylase enzyme production in the cheaper medium. In this research work, to study the enzyme activity, two assays that are Iodine assay and DNSA assay techniques are used. Alpha-amylase starch degrading capability will also be discussed on the maize kernels.

MATERIAL AND METHOD

Preservation of bacterial strain

Bacillus subtilis, gram positive bacteria was used in the present study. For the preservation, the bacterium was grown on nutrient agar plate and for growth it was sub-cultured on the slant containing basal medium of following composition (g/L):10:00 beef extract, 5:00 NaCl, 12:00 Nutrient Agar. The initial pH and temperature of the medium was adjusted to 7.0 with 0.1N NaOH and 25^{0}C respectively. Its cultivation was done with 3ml, 10ml, 30ml, and 100ml of medium in Erlenmeyer flask. Typical culture and morphological characteristics were observed for *Bacillus subtilis* in the present study. Growth of the bacteria and amylase production was measured at 0hour, 24 hours, 48 hours and 72 hours.

Growth Kinetics

The basal medium used for the cultures was composed (g/L) of 5.00 peptone, 1.50 yeast extract, 1.50 beef extract, 5.00 NaCl. The pH 7.4±0.2 and temperature 25^{0}C (after sterilization) were adjusted. Growth curve and secretion of α-amylase enzyme were studied in basal medium of 100ml with 10% inoculum taken from 30ml culture, in 250ml Erlenmeyer flask which was incubated up to 72 hours. In all levels of culture medium, inoculum was always kept uniform at Abs 600 nm at different levels of the inoculum used (3 ml, 10 ml , 30 ml, & 100 ml). The flasks were analyzed for growth (A_{600}). The biomass (pellet weight) of the bacteria was measured by centrifugation at 8000rpm for 20 minutes at 15^0 C for different level at different period of their growth. The cell-free supernatant was used as the enzyme source.

2.3 Measurement of α-amylase activity

In the present study two methods i.e., Iodine assay and DNSA assay were used to determine the liquefying and saccharifying activity of the alpha enzyme.

Enzymatic activity was measured by starch as substrate. 2ml. of 0.2% starch was added with 1ml of culture supernatant and allowed to react for 40^{0}C for 10 minutes at room temperature. After incubation, the reaction was stopped by the addition of this reaction mixture to 5ml of iodine reagent (Wilson and Ingeldew, 1982) and absorbance was measured in a UV spectrophotometer (Hitachi) at 620nm against control. One unit (μ) is defined as the amount of enzyme which hydrolyzes 0.1mg of starch in a 10 minute reaction at 40^{0}C, when 4mg of starch is present.

Amylase activity was calculated by (1)

$$amylaseunits/ml = \frac{(\text{Abs control} - \text{Abs test})}{\text{Abs control}} \times 40D$$

Where, D= Enzyme dilution factor and 40 represents 4 mg of starch present in reaction tube times 10.

A total of 0.5ml enzyme solution and 0.5ml soluble starch (1%v/v) in 0.02 M phosphate buffer (pH 6.9) were mixed and allowed to react at 30^{0}C for different periods: 0 minute, 3 minutes, 6 minutes, 9 minutes, 10 minutes, 12 minutes, 15 minutes, 20 minutes and 40 minutes. To stop the reaction, 2ml of DNSA reagent was added to the reaction mixture. (The DNSA reacts with the reducing sugars released by the amylase). The reaction mixture was heated for 5 minutes in a boiling water bath and

then kept in cold water for cooling and incubated at normal room temperature for 20-60 minutes Reducing sugar was determined by comparing absorption at 540nm of the assay solution to a standard curve of maltose solution (0.2-1 mg/ml). One unit of amylase activity was defined as the released of 1µmol reducing sugar from the soluble starch per minute.

Activity of α-amylase to hydrolyze maize starch

α-amylase extracted form *Bacillus subtilis* was used to hydrolyze the maize kernel starch into maltose, an applicability for food uses was demonstrated. 10ml of supernatant enzyme extracted from 96h culture growth of *Bacillus subtilis* in a 100ml scale growth was added to equal amount of uncrushed, crushed and sieved maize kernels in glass tubes. The kernels were heated with or without enzyme in boiling water bath for 5 minutes and incubated for overnight at room temperature. The reaction mixture was incubated in room temperature. The overnight incubated sample was centrifuged at 8000rpm for 20 minutes in cooling centrifuge. The suspension was then assayed for reducing sugar released using Bernfield's method with DNSA reagent. The control was a tube with the DNSA reagent and with distilled water instead of enzyme extracted and absorbance was measured at 540nm.

RESULT AND DISCUSSION

Characterization of bacterial strain

The *Bacillus subtilis* was confirmed as gram positive bacteria. The size of colony was 0.1mm in diameter was observed on nutrient agar medium. The shape of colonies observed were either rounded or eclipsed on the nutrient agar. Bacteria colonies were observed with creamish white in colour. The strain *Bacillus subtilis* possessed the ability to produce α-amylase and hydrolyze starch.

Growth Kinetics

Growth kinetic of the strain *B.subtilis was* done with respect to α-amylase production in the basal medium with pH 7.0 at 25˚C. The lag phase for *B.subtilis* was very short (4 hr), after that it showed exponential growth up to 32 hrs followed by stationary phase till 48 hrs and from 54 hrs it started to decline. The enzyme levels tend to decline either due to the increase in the glucose concentration, or due to the rise of protease levels in the environment or due to the drastic change in pH levels.

A culture yield of 11g/100 ml growing cells in exponential phase, were obtained after 28h grow in 100ml working volume. The specific growth rate of increased cell biomass; 0.036 h^{-1} was determined between 6 to 28 h. The mean doubling time for biomass for the culture was 22 h. The viable count showed differences in both nutrient agar and plate count absorbance observed was higher in plate count agar media and count observed was 10-14 $\log_{10}$ cfu/ml in both media. This difference was occurred due to difference in aerobicity and glucose level. Thus, the specific growth rate (µ) 0.355 h^{-1} was obtained in plate count agar medium. The amylase production pattern in this organisms also indicates that the induction of the amylase took place during the log phase and the maximum yield is obtained, after 48h of incubation, in the mid stationary growth phase which is probably due to release of all the intracellular fractions of the enzyme during the stationary phase because of cell lysis. Fig.4,5

Measurement of α-amylase activity

In the present study, α-amylase has proven to hydrolyze the starch. Iodine assay was performed in *Bacillus subtilis* growth in basal medium for liquefying action, showed that the α-amylase production in 100ml peaked (25.56 U/ml) at 24h and was found to decline gradually up to 96 hours 17.78 U/ml. figure 6 and in 30ml peaked (35U/ml) at 72h and was found to decline after that. It was reported that α-amylase production in lower volume as in 30ml does not reduce to the extent as in higher volume as 100ml. The reasons may be excretion of other metabolites like protease in higher level in higher volume of broth and heterologous proteins like amylase are often rapidly degraded in the presence of such extracellular protease. It was observed that maximum alpha amylase production by *Bacillus subtilis NCIM 2479* occurred when cell population reached the peak. DNSA assay performed in *Bacillus subtilis* growth in basal medium for saccharifying action showed activity and specific activity of enzyme reduced when the reaction time increased for the enzyme. The reason behind this fact may be secretion of other substances by organism which degrade the amylase protein and reduce the activity and hence its specific activity.

Activity of α-amylase to hydrolyze maize starch

The maize kernels with alpha amylase were used for the hydrolysis of starch. The extent of saccharification was demonstrated on releasing maltose as reducing end group and smallest crushed grains released the maltose in higher amount in compare to uncrushed grain which released low amount of maltose as reducer.

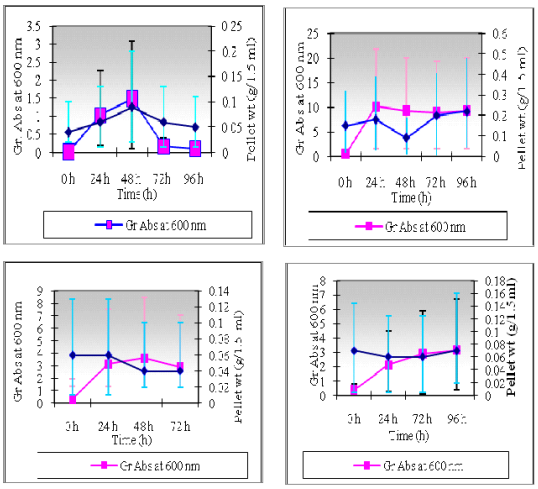

Fig.1 Growth absorbance and fresh cell weight (centrifuged pellet wt.1.5 ml NB broth) of *Bacillus subtilis* growth in 3ml(a), 10ml(b), 30 (c) and 100 ml (d) of Nutrient broth medium

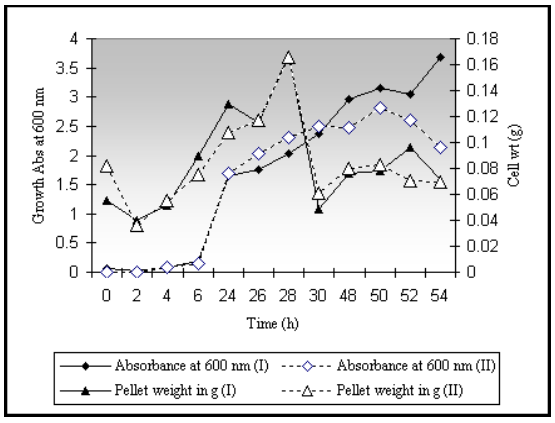

Fig.2 Growth of *Bacillus subtilis* Absorbance at 600 nm v/s cell (fresh) wt in duplicate flasks (I & II).

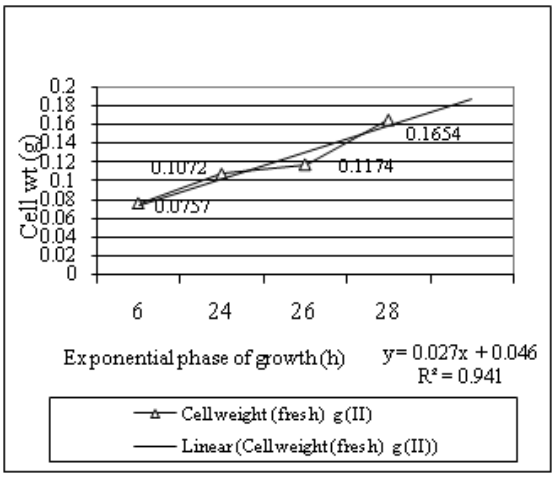

Fig.3 Cell weight in exponential phase II

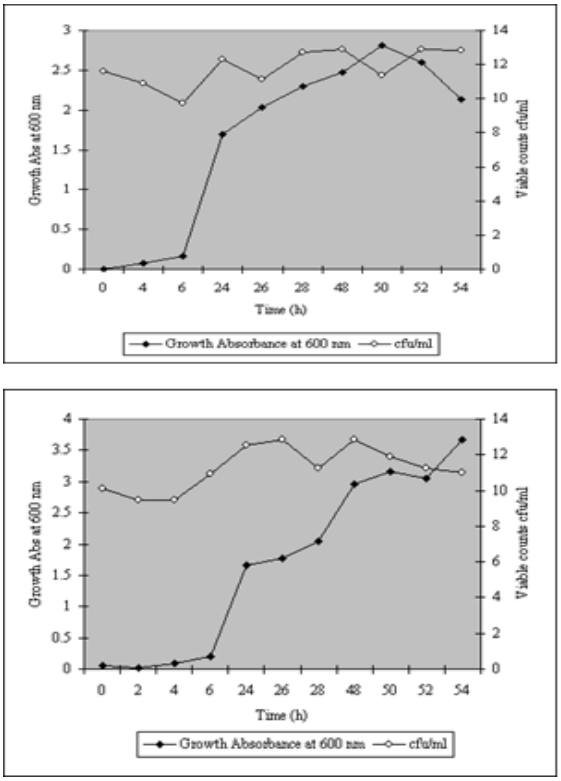

Fig. 4 (a & b) Viable counts (cfu/ml) of *Bacillus subtilis* on nutrient agar (a) and plate count agar (b) media

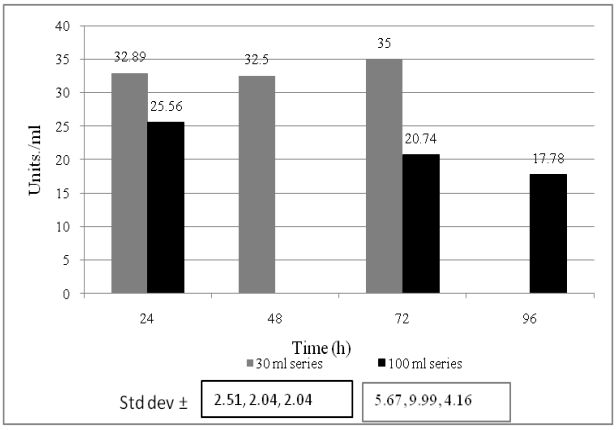

Fig. 5 Enzyme α-amylase (assayed as liquefying activity) secreted during growth of *B. subtilis* in two different working volumes of the Nutrient broth.

CONCLUSION

The *Bacillus subtilis NCIM 2479* produced high level of α-amylase with characteristics suitable for application in starch processing and other food industries. The production process can be commercialized after further optimization for enhanced enzyme production. It will be beneficial for all living life. Even this enzyme can be used for polythene degradation.

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